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STUDIES ON SYNTHETIC MEDIUMS

I. STUDY OF THE CHARACTERISTICS OF SOME BACTERIA ON A SIMPLE SYNTHETIC MEDIUM

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The study of the metabolism of bacteria has made very little progress until the present time, because it is impractical to study the end-products of any reaction without knowing the chemical composition of the raw materials. The basis of most bacterial culture mediums in the past has been beef infusion or extract, and peptone with the addition of agar to solidify it. It is usually stated that the agar does not add anything to the food of the bacteria, but merely gives a convenient neutral solid. This is not true. It has been found in this laboratory that B. typhosus will grow on a medium made of agar alone, which was previously extracted a number of times with water. Peptone is a mixture, obtained from the digestion of protein with pepsin and hydrochloric acid, and does not represent a chemical entity. In order, then, to attack the problem of bacterial metabolism, it is necessary to secure a culture medium of perfectly definite chemical composition, which can always be duplicated.

Uschinsky[1] had this in mind when he proposed his synthetic medium. Most synthetic mediums, since his time, have been some modification of his formula. However, it is possible to grow bacteria in a medium much simpler than Uschinsky's. Aside from the fact that the medium must contain sufficient nitrogen, carbon, etc., in a form in which they can be assimilated by the bacteria, the medium must be nearly isotonic with bacterial protoplasm and must have and maintain a favorable hydrogen-ion concentration.

It has been repeatedly pointed out that small variations in the hydrogen-ion concentration play a large rôle in biological processes. It has been shown in many researches on the action of enzymes that in order that satisfactory results be obtained, it is necessary to use solu-

Received for publication, July 20, 1918.

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[1] Centralbl. f. Bakteriol., I, O., 1893, 14, p. 346.

tions that are prepared with a view to preventing sharp variations in the hydrogen-ion concentration. The same holds true for the study of the growth of microbes, in whose metabolism it has been shown that enzymes have a prominent place. In these experiments, much attention has been given to preparing the culture medium so that it would prevent sharp changes in the hydrogen-ion concentration, and to this end a solution as heavily buffered as possible, consistent with osmotic requirements, was used. It is believed that this type of culture medium offers the most satisfactory means for the study of bacterial growth and metabolism, since it admits of prolonged growth without undue change in conditions. Especially is this necessary with organisms which have closely limited zones of optimum growth.

Clark and Lubs[2] in their recent work have indicated broadly the importance of these considerations. In working with synthetic medium of simple composition, Doryland[3] apparently overlooked this aspect of the problem, for in his paper no attention is given to this phase of the work. It is true that his medium, neutralized to phenolphthalein (p_H-8.5) contained about 15 gm. of mixed phosphates per liter, so that the solutions used by him were probably heavily enough buffered.

An attempt was made in these experiments to keep the osmotic pressure of the medium about that of mammalian blood serum, that is, isotonic with M/6 NaCl. The medium used contained alkali equivalent to 0.218 mols which, when neutralized with acid, yielded a salt concentration of 0.218 molar which when we remember that phosphates are only about 65% dissociated gives a solution almost isotonic with M/6 NaCl. In Doryland's experiments a much greater osmotic pressure was maintained. In his work he used a solution containing nearly 40 gm. of salts per liter which would probably be isotonic with M/2 or M/3 NaCl. It may probably be due to this unfavorable osmotic environment that many of the organisms he worked with did not grow.

A medium was prepared having the simplest composition consistent with these requirements. In the first experiments, the medium was made in hydrogen-ion concentrations varying from 10^{-5}-10^{-8} N. It consisted of phosphoric acid, acetic acid, ammonia and potassium and sodium hydroxid in such proportions as to give the desired hydrogen-

[2] Jour. Bacteriol., 1917, 2, p. 1, etc.

[3] Ibid., 1916, 1, p. 135.

ion concentration. The medium in its final form was of the following composition:

$$\left.\begin{array}{l}129.5 \text{ c c of molar } H_3PO_4 \\ 18.8 \text{ c c of molar } CH_3COOH \\ 17.8 \text{ c c of molar } NH_4OH\end{array}\right\} \text{diluted to 500 c c.}$$

and to this was added 10 c c each of 0.01% $CaCl_2$, 0.01% $FeCl_3$ and 0.01% $MgSo_4$ and sufficient 2 molar alkali (equal mols of NaOH and KOH) to give the proper hydrogen-ion concentration. The amounts used are given in Table 1.

TABLE 1

AMOUNTS USED IN THE PREPARATION OF THE MEDIUM

PH	C c of Stock	C c of 2 Molar Alkali	C c Final Dilution
5	500	62.5	1,000
6	500	85	1,000
7	500	100	1,000
8	500	125	1,000

It was not feasible to make any lower hydrogen-ion concentrations, as when the solution becomes alkaline the phosphates are preciptated out. The solution containing the phosphoric, acetic acids, ammonia and alkali was diluted to 1,000 c c, after the addition of 10 c c each of 0.01% $CaCl_2$ $MgSo_4$ and $FeCl_3$. The medium was water clear, and after being tubed in sterile tubes was sterilized by steam on 3 successive days, for 15 minutes. The hydrogen-ion concentration was determined after each sterilization and in no case did it change.

This medium was inoculated with a group of organisms which were chosen as representatives of the different classes of bacteria as grouped in Chester's Manual. No streptococci were used, as it was not feasible to carry streptococci along with the other organisms. The organisms were planted at first merely with the idea of seeing how many would grow in a medium of this composition. The organisms were planted in duplicate by each of 2 workers, and one set of each was used for recording growth, while the other was used for determining the change in hydrogen-ion concentration. After 24 hours 3 loopfuls of the culture were placed on the surface of an agar slant and growth recorded as positive or negative after 24 hours. The 1 culture was incubated for a week just as it was, while the second one, after the transplant to agar was made, was divided into 2 portions — 1 was used for the hydrogen-ion concentration determination after 24 hours, while the second portion was incubated for 1 week, when transplants were again made and the hydrogen-ion concentration determined. The hydrogen-ion concentration was obtained by adding Clark and Lubs[2] indicators to the cultures and comparing the colors obtained with a set of standards, prepared according to Sörensen, treated with the same indicators. To each culture was added at least 3 suitable indicators. The results of the experiments are found in Table 2.

TABLE 2

GROWTH ON SYNTHETIC MEDIUM

Name of Organism	Medium with a Reaction of 10^{-5N}					Medium with a Reaction of 10^{-6N}				
	Growth		Final PH		Growth after 8 Days	Growth		Final PH		Growth after 8 Days
	24 Hrs.	8 Days	24 Hrs.	8 Days		24 Hrs.	8 Days	24 Hrs.	8 Days	
1. B. mucosus	+	+	5.5	7	Slight	+	+	6.5	7	Heavy
2. M. tetragenus	—	—	5	5	—	—	—	6	6	—
3. B. subtilis	+	+	6	7	Slight	+	+	6.5	7	Slight
4. B. coli communior	+	+	6	7	Slight	+	+	6.5	7	Heavy
5. B. pyocyaneus	+	+	6	7	Moderate, pigment	+	+	6	7	Heavy, pigment
6. Staph. pyogenes albus	—	—	5	5	—	—	—	6	6	—
7. B. enteritidis	+	+	5.2	7	Heavy	+	+	over 6	7	Moderate
8. B. prodigiosus	+	+	5.2	6.5	Slight	+	+	over 6	7	Slight, pigment
9. B. megatherium	+	+	5.2	6	Slight	+	+	over 6	7	Slight
10. M. candicans	—	—	5	5	—	—	—	6	6	—
11. B. fecalis alkaligenes	+	—	5.2	6.5	—	+	+	over 6	7	Slight
12. B. anthracoides	+	+	5.2	6.5	Slight	+	+	over 6	7	Slight
13. M. catarrhalis	—	—	5	5	—	—	—	6	6	—
14. B. enteritidis—Gaertner	+	+	5.2	6	Slight	+	+	over 6	7	Slight
15. Staph. pyogenes aureus	—	—	5	5	—	—	—	6	6	—
17. B. mirabilis	+	+	5.2	7	Slight	+	+	7	7	Slight
18. M. citreus	+	+	6	7	Slight	—	+	7	7	Slight
19. B. aerogenes	+	+	5.2	7	Moderate	+	+	over 6	7	Moderate
20. B. cloaceae	+	+	5.2	7	Heavy	+	+	7	7	Heavy
21. B. hemorrhagicus	—	—	5	5	—	—	—	6	6	—
23. B. fluorescens liquefaciens	+	+	5.2	7	Moderate	+	+	7	7	Moderate
24. B. acidi lactici	+	+	5.2	7	Heavy	+	+	over 6	7	Heavy
25. B. buccalis	+	+	5.2	7	Slight	+	+	over 6	7	Slight
27. B. cholerae	+	+	5.2	7	Moderate	+	+	over 6	7	Moderate
28. B. viridans	+	+	5	7	Moderate	+	+	over 6	7	Moderate
29. Sp. rubrum	—	—	5	5	—	—	—	6	6	—
30. B. dysentery—Kruse	+	+	5	7	Slight	+	+	over 6	7	Slight
31. Msp. cholerae	+	+	5.2	7	Slight	+	+	over 6	7	Slight
32. B. lactis aerogenes	+	+	5.2	7	Moderate	+	+	over 6	7	Heavy
35. M. roseus	—	—	5	5	—	—	—	6	6	—
36. Sarc. roseus	—	—	5	5	—	—	—	6	6	—
37. B. capsulatus	+	+	5.2	7	Heavy	+	+	6	7	Heavy
38. Sarc. lutea	+	—	5.2	7	Moderate, produces pigment	+	+	over 6	7	Slight, pigment
39. B. dysenteriae—Shiga	+	+	5.2	7	Slight	+	+	7	7	Slight
40. B. erythrogenes	—	—	5	5	—	—	—	6	6	—
41. B. violaceus	+	+	6	7	Slight, pigment	+	+	7	7	Heavy
42. B. proteus vulgaris	..	..	...	...	....	..	..	....	...	....
43. B. typhosus	..	..	. .	...	....	..	..	....	...	....

Two more points remained to be proven before the statement could be made that the medium was a good medium on which to grow these organisms: (1) that the organism would remain alive for long periods in the medium; (2) that the organism after growing on the synthetic medium for long periods of time had the same characteristics as before.

TABLE 2—*Continued*

GROWTH ON SYNTHETIC MEDIUM

Medium with a Reaction of 10^{-7N}					Medium with a Reaction of 10^{-8N}				
Growth		Final PH		Growth after 8 Days	Growth		Final PH		Growth after 8 Days
24 Hrs.	8 Days	24 Hrs.	8 Days		24 Hrs.	8 Days	24 Hrs.	8 Days	
+	+	7	7	Heavy, flocculent sediment, greasy ring around sides of tube at top	+	+	8	8	Heavy
+	—	7	7	Moderate	—	—	—	—	—
+	+	7	7	Moderate, a slight turbidity persisting	+	+	8	8	Slight
+	+	7	7	Heavy, slight sediment, greasy growth around sides of tube at top	+	+	8	8	Moderate
+	+	7	7	Heavy, much sediment, pigment in 24 hours	+	+	8	8	Heavy, pigment
+	+	7	7	Slight	—	—	8	8	—
+	+	7	7	Heavy, slight sediment	+	+	8	8	Heavy
+	+	7	7	Moderate, turbidity evenly distributed, pigment	+	+	8	8	Slight
+	+	7	7	Heavy, marked turbidity, much sediment	+	+	8	8	Slight
—	—	7	7		—	—	8	8	—
+	+	7	7	Moderate, slight sediment	+	+	8	8	Heavy
+	+	7	7.5	Heavy, marked turbidity, sediment	+	+	8	8	Slight
—	—	7	7		—	—	8	8	—
+	+	7	7	Moderate, sediment	+	+	8	8	Slight
+	+	7	7	Slight	—	—	8	8	—
+	+	7	7	Moderate	+	+	8	8	Moderate
+	+	7	7	Moderate, pigment developed in 8 days	+	+	8	8	Slight
+	+	7	7	Moderate, much flocculent sediment, slight turbidity	+	+	8	8	Moderate
+	+	7	7	Heavy, pellicle	+	+	8	8	Heavy
—	—	7	7		—	—	8	8	—
+	+	7	7	Green pigment, heavy pellicle in 24 hours, slimy sediment	+	+	8	8	Moderate
+	+	7	7.5	Heavy, marked turbidity, slight sediment	+	+	8	8	Heavy
—	+	7	7	Moderate, slight turbidity	+	—	8	8	Slight in 24 hours, negative in 8 days
+	+	7	7	Moderate, moderate turbidity, some sediment	+	+	8	8	Heavy
+	+	7	7.5	Moderate, greenish pellicle in 24 hours, falls to bottom of tube	+	+	8	8	Heavy, pigment
+	+	7	7	Slight, slight sediment	+	—	8	8	Slight for 24 hours, negative at 8 days
+	+	7	7	Slight, flocculent sediment	+	+	8	8	Slight
+	+	7	7	Slight, slight sediment	+	+	8	8	Moderate
+	+	7	7	Heavy, much flocculent sediment	+	+	8	8	Heavy
+	+	7	7	Slight, produced pigment	—	—	8	8	—
+	+	7	7	Heavy, marked turbidity, produces pigment	—	—	8	8	—
+	+	7	7	Moderate, flocculent sediment, greasy ring around sides of tube at top	+	+	8	8	Heavy
+	+	7	7	Slight, pigment produced	+	—		8	Negative at 8 days, slight in 24 hours
+	+	7	7	Slight	—	—		8	—
+	+	7	7	Slight	—	—		8	—
+	+	7	7	Moderate, moderate turbidity, pigment	+	+		8	Moderate
+	+	7	7.5	Heavy, slimy sediment					
+	+	7	7	Slight turbidity, slight sediment					

In order to carry out these observations, the organisms were carefully studied before and after they were planted on the synthetic medium. Since the greater number of organisms grew on the medium having an initial hydrogen-ion concentration of 10^{-7} and also gave better growth on this medium, this concentration was used. The medium had the following composition:

129.5 c c of molar H_3PO_4	12.79 gm.	diluted up to give one liter.
18.8 c c of molar CH_3COOH	1.13 gm.	
17.8 c c of molar NH_4OH	0.62 gm.	
100 c c of molar NaOH	4.0 gm.	
100 c c of molar KOH	or 5.6 gm.	
10 c c of 0.01% $FeCl_3$	0.001 gm.	
10 c c of 0.01% $MgSO_4$	0.001 gm.	
10 c c of 0.01% $CaCL_2$	0.001 gm.	

The medium, as made up, was made from stock solutions which had been standardized against standard acid and alkali, but as this is not feasible in all laboratories, the formula is also given so that the various acids and salts may be weighed out.

The organism was inoculated into 2 tubes of the synthetic medium, having this composition and a hydrogen-ion concentration of 10^{-7} N. The tubes were incubated for 24 hours and 3 loopfuls from 1 tube were placed on the surface of an agar slant. The second tube was divided into 2 portions, 1 portion being used to determine the hydrogen-ion concentration, the other portion being incubated for a longer time for the same purpose.

TABLE 3

Summary of Growth at Different Initial Hydrogen-ion Concentration

Initial P_H	No. Planted	No. Grew in 24 Hrs.	No. Grew in 8 Days	No Growth
5	36	26	24	10
6	36	25	26	10
7	38	34	34	3
8	36	25	23	10

The agar slant was incubated for 24 hours and then the culture was studied in the following manner:

1. Morphology:
    - Staining.
    - Spore formation.
    - Flagella.
    - Capsules.
2. Biologic Characteristics:
    - (*a*) Plate cultures on agar.
        - Deep and surface colonies for form, size, margin, surface, texture, color and consistency.
    - (*b*) Agar tube culture.
        - Slants and streaks for same characters as colonies on plates.

TABLE 4

CHARACTERISTICS AFTER GROWTH ON SYNTHETIC MEDIUM

No.	Change after		
	24 Hours	8 Days	1 Month
1	Peptonization of milk complete 24 hours sooner than before growth on synthetic medium	Same as 24 hours	
2	Growth same as before	Did not grow	Did not grow
3	Coagulation; reduction; peptonization of milk complete in 3 days as compared to 6 before planting in synthetic medium	Same as 24 hours	Complete in 2 days
4	Coagulation of milk more rapid; peptonization complete in less time	Same as 24 hours	
5	Gelatin liquefied more slowly; peptonization of milk more rapid	Same as 24 hours	Same as 24 hours for milk and gelatin; pigment produced very slowly; thermal death point changed from 15-30 min. at 63 C.
6	Altho this organism should liquefy gelatin it did not till after it had grown on the synthetic medium	Same as 24 hours	Did not grow
7	Same as before	Same	Same
8	Pigment appeared more quickly on potato; milk action more rapid; gelatin liquefaction slower	Same as 24 hours	Same as 24 hours
9	Peptonization of milk more rapid; liquefaction of gelatin slower	Same as 24 hours	Same as 24 hours
11	Same as original	Same as the original culture	
12	Peptonization of milk more rapid; liquefaction of gelatin slower	Same as 24-hour culture	
14	Same as the original culture in all respects (24, 8 days, 1 month)		
15	Same as the original culture		Did not grow
17	Same as the origianl culture in all respects (24, 8 days, 1 month)		
18	Same as the original culture in all respects (24, 8 days, 1 month)		
19	Same as the original culture in all respects (24, 8 days, 1 month)		
20	Milk formed acid and coagulated more quickly; original did not liquefy gelatin in 7 days; after growth on synthetic medium, liquefied in 3 days	Same as 24 hours	
23	Same as the original culture in all respects (24, 8 days, 1 month)		
24	Same as the original culture in all respects (24, 8 days, 1 month)		
25	Did not grow	Same as original culture in all respects	
27	Coagulation of milk and peptonization more rapid than original	Same as 24-hour culture	
28	Same as the original culture in all respects (24, 8 days, 1 month)		
30	Same as the original culture in all respects (24, 8 days, 1 month)		
31	Liquefaction of gelatin slower	Same as 24-hour culture	
32	Same as the original culture in all respects (24, 8 days, 1 month)		
36	Same as the original culture in all respects (24, 8 days, 1 month)		
37	Peptonization of milk more rapid	Same as 24-hour culture	
38	Same as original culture in all respects		
39			
40			
41			
42			
42			

  - (*c*) Potato cultures—being especially careful to note production of gas, odor or discoloration.
  - (*d*) Gelatin.
    Both stabs and streaks—time of liquefaction especially being noted.
  - (*e*) Bouillon—turbidity, surface growth, odor and sediment.
  - (*f*) Litmus milk—reaction, coagulation, digestion of casein, splitting of lactose, reduction of litmus, odor.

3. Biochemical Factors:
   - (*a*) Production of enzymes.
     Sugar splitting with formation of acid and gas, coagulating and diastatic.
   - (*b*) Production of indol and phenol.
   - (*c*) Production of acid or alkali.
   - (*d*) Reduction of nitrates to nitrites.
   - (*e*) Reduction of litmus.
4. Pathogenicity: Reported in another paper.

The organisms were studied, as stated, after growing on the synthetic medium for 24 hours, at which time transplants were made to agar. The hydrogen-ion concentration was determined on ½ of the contents of another tube. At the end of 8 days, one transplant was made to agar and a second one from the same tube by transferring, with a sterile pipet, 0.1 c c of the culture to another sterile tube of the synthetic medium. The culture in this tube was transplanted thereafter every 4 days for a period of at least 1 month. The hydrogen-ion concentration was determined on the tube which had incubated for 8 days. At the end of the month, transplants were made to agar and the organisms studied culturally according to the scheme presented. In addition, thermal death points were determined on the organisms which had grown for a month on the synthetic medium. The changes which took place are given in Table 4.

From the results it is apparent that the organism grown on the synthetic medium had all the necessary elements for their growth. None of the characteristics were lost and in the case of the peptonization of milk the process seemed to be hastened by growth on synthetic medium. This may be merely a question of favorable hydrogen-ion concentration for the process; just as the retarding of the time of liquefaction of gelatin may be a question of unfavorable hydrogen-ion concentration. Whether or not these surmises are correct can only be proven by further work which is under way. The medium in its present form is suitable for the growth of most saprophytic organisms and also for some of the facultative parasitic organisms, and it is hoped that the medium can be slightly modified so that the obligate parasites will grow in it. A solid medium of known composition will be the next step in the investigation.